Antimicrobial susceptibility and virulence-associated genes in *Campylobacter* isolates from milk and wastewater in Hatay, Turkey

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ABSTRACT: Campylobacter is one of the most common causes of bacterial foodborne diseases throughout the world. This study was conducted to determine the prevalence, antimicrobial resistance and virulence of Campylobacter isolates of raw cow’s milk and cattle slaughterhouse wastewater samples in Hatay, Turkey. A total of 114 raw milk and 78 wastewater samples were analyzed for the identification of *C. jejuni*, *C. coli*, and *C. lari* by multiplex PCR. The overall prevalence of Campylobacter was found to be 7.2%, of these isolates, 85.7% were identified as *C. jejuni* and 14.2% as *C. coli*, but *C. lari* was not detected in the study. The cdtA and cadF genes were present in 66.6% and 41.6% of *C. jejuni* isolates tested, respectively, but wlaN gene was not found in any of the isolates. Results of antimicrobial resistance analysis showed that 71.4% of the isolates were resistant to erythromycin, 64.2% to tetracycline, and 57.1% to ciprofloxacin. Overall, 8 of 14 Campylobacter isolates (57.1%) showed a multidrug resistance.

Key words: antimicrobial resistance, *Campylobacter*, milk, wastewater.

INTRODUCTION

Campylobacters are zoonotic, and main reservoirs of them are wild and domestic animals, especially intestinal tract of birds. Poultry meat, and its products are considered as the common source of *Campylobacter* infection in humans (PEZZOTTI et al., 2003; PÉREZ-BOTO et al., 2010; DUARTE et al., 2014; BOLTON, 2015; WIECZOREK & OSEK, 2015; ZHONG et al., 2016), but also cattle may play an important role for human campylobacteriosis. There are cattle-related outbreaks both in the United States and Europe indicated that raw milk and dairy products are the second most frequently sources of infection (WHO, 2013). The most common symptom in campylobacteriosis in humans is acute gastroenteritis, but the infection is sometimes complicated with Guillain-Barré syndrome. Campylobacters are usually isolated from contaminated poultry meat, raw milk, and water (MOORE et al., 2001; DOMÍNGUEZ et al., 2002; PEZZOTTI et al., 2003; YANG et al., 2003; WHYTE et al., 2004; HUSSAIN et al., 2007; BARDÓN et al., 2011).

*Campylobacter jejuni* and *Campylobacter coli* are most important species commonly associated with foodborne gastroenteritis. *Campylobacter* species are gram-negative, motile, termophilic and capnophilic bacteria that need microaerobic conditions for optimal growth. The minimal infective dose of *C. jejuni* is very low. It means that *C. jejuni* has a high virulence and very small numbers of bacteria cells could cause infection in humans (YANG et al., 2003;
The virulence of Campylobacter species is associated with flagellar motility, adhesion, invasion and production of cytolethal distending toxins (ROZYNEK et al., 2005; MARTÍNEZ et al., 2006; WIECZOREK & OSEK, 2008; RIPABELLI et al., 2010; GONZALEZ-HEIN et al., 2013; DI GIANNATALE et al., 2014; BOLTON, 2015). Fluoroquinolones and macrolides are frequently used in the treatment of campylobacteriosis, so that an increased level of resistance to these antimicrobials is observed in Campylobacter (PEZZOTTI et al., 2003; PÉREZ-BOTO et al., 2010; QIN et al., 2011; DI GIANNATALE et al., 2014; WIECZOREK et al., 2015; ZENDEHBAD et al., 2015). Since the poultry and poultry meat are the most important sources of Campylobacter infections, many authors have studied the prevalence of Campylobacter on poultry in different countries (ZANETTI et al., 1996; DOMINGUEZ et al., 2002; PEZZOTTI et al., 2003; YANG et al., 2003; WHYTE et al., 2004; HUSSAIN et al., 2007; BARDOŇ et al., 2011; ZENDEHBAD et al., 2015; ZHONG et al., 2016). Also, recently there are a lots of reports regarding isolation of Campylobacter from raw milk and slaughter animals (GONZALEZ-HEIN et al., 2013; DI GIANNATALE et al., 2014; WYSOK et al., 2015a; WYSOK et al., 2015b; KALMUS et al., 2015; BERTASI et al., 2016; KASHOMA et al., 2016). But, searches in order to get more information about virulence mechanism and antimicrobial resistance profile of Campylobacter from these sources (raw milk, wastewater) are still limited. For this purpose, antimicrobial resistance and virulence genes of Campylobacter isolates recovered from raw milk and slaughterhouse wastewater samples were investigated in this study.

MATERIALS AND METHODS

A total of 114 raw cow’s milk and 78 wastewater samples were collected from three different dairy farms and two cattle slaughterhouses in rural areas of Hatay province at a distance of 60-70 kilometers. All samples were aseptically taken at different times and were transported to the laboratory on the day of collection under cold chain. In the next step, 25 mL from each sample was placed into a sterile polyethylene bag containing 100 mL of Campylobacter Enrichment Broth (Base) (LAB135, Lab M, UK) supplemented with CAT (Cefoperoxzone-Amphotericin B-Teicoplanin; FD145, Himedia, India) and incubated for 48 hours at 42°C under microaerobic conditions with using microaerophilic kits (Anaer Pack-MicroAero, MGC, Japan). Then, a loopful from all pre-enriched samples was streaked onto selective solid agar, Campylobacter Blood Free Medium Base Bolton (mCCDA, Biolife, Italy) supplemented with CCDA (Cefoperoxzone-Amphotericin B; SR0155, Oxoid). The plates were incubated at 42°C for 24-48 hours under microaerobic conditions (HUNT et al., 2001). Presumptive Campylobacter colonies were selected for further identification by PCR.

The isolates were identified to the genus/species level by multiplex PCR. C. jejuni ATCC 29428 (Microbiologics, USA) and C. coli ATCC 43478 (Microbiologics, USA) were used as positive controls in molecular analysis. Four pairs of primers were used for amplifying the 16S rRNA gene specific for the genus Campylobacter (LINTON et al., 1996), the ask gene specific for C. coli (LINTON et al., 1997), the glyA gene specific for C. lari (WANG et al., 2002), and the cJ0414 gene specific for C. jejuni (WANG et al., 1992). The DNA extraction from the isolates was performed using a Bacterial DNA Extraction kit (Nucleic Acid Extraction Kit, GF-1, Vivantis, Malaysia), following the kit manufacturer’s instructions. For DNA amplification, PCR protocol previously described by WANG et al. (2002) was used in the study.

In order to detect some virulence genes (cadF, cdtA, wlaN) in the isolates confirmed as C. jejuni, specific primers described by KONKEL et al. (1999), HICKEY et al. (2000), LINTON et al. (2000) were used. All primers used in the study are shown in table 1. The PCR mixture and amplification of virulence genes were carried out according to DATTA et al. (2003), as previously described. Termalcycling conditions consisted of 30 cycles of 94°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min. The annealing temperature was set to 46°C for cadF, wlaN and to 49°C for cdtA.

All isolates were examined for antimicrobial resistance by determination of minimum inhibitory concentrations (MICs) on Mueller-Hinton agar (M1084, Himedia, India) by E-test (BAKER et al., 1991). The MICs were determined by using MIC test strips (Liofilchem, Italy) containing concentrations of the following antimicrobials: ciprofloxacin (0.002-32 mg/L), erythromycin (0.016-256 mg/L), and tetracycline (0.016-256 mg/L). The MIC breakpoints were evaluated according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2014). The isolates resistant to three antimicrobials at the same time were considered as multidrug resistant.
RESULTS AND DISCUSSION

In the current study, 114 raw milk and 78 cattle wastewater samples were analyzed for the presence of Campylobacter. A total of 14 (7.2%) Campylobacter isolates were obtained from 192 samples. Of the raw milk samples, 6 (5.2%) were found to be contaminated with Campylobacter jejuni. Overall, 6 (7.6%) C. jejuni and 2 (2.5%) C. coli strains were recovered from the wastewater samples. Results of the study demonstrated that wastewater samples were more frequently contaminated with Campylobacter than raw milk. The most prevalent species was C. jejuni with a rate of 85.7% (12/14). The remaining of isolates (14.2%, 2/14) were confirmed as C. coli by PCR (Figure 1). In the study, C. lari was not detected.

Overall, 9 C. jejuni strains were found to have virulence genes. It was found that C. jejuni isolates harbored mainly cdtA gene (66.6%, 8/12) whereas the wlaN gene associated with Guillain-Barré syndrome was not present in any of the isolates. Another virulence gene, cadF which encodes extramembrane proteins involved in adhesion and invasion, was detected in 5 of 12 (41.6%) analyzed isolates.

Results of antimicrobial resistance analysis showed that 10 of 14 isolates (71.4%) were resistant to erythromycin, 9 (64.2%) to tetracycline and 8 (57.1%) to ciprofloxacin. It was found that 8 (57.1%) of the 14 Campylobacter isolates were resistant to all three antimicrobials at the same time. The most prevalent resistance profile observed in C. jejuni was erythromycin (66.6%), followed by tetracycline (58.3%) and ciprofloxacin (50%). C. coli isolates were resistant to all three antimicrobials (Table 2).

When we examined some studies conducted in Turkey and in other countries, campylobacters were frequently isolated from poultry and poultry meat at high levels. Similarly, C. jejuni was isolated as the most common species in many studies, followed by C. coli (ZANETTI et al., 1996; ELMALI & YAMAN, 2004; WHYTE et al., 2004; HUSSAIN et al., 2007; BARDOŇ et al., 2011; WIECZOREK & OSEK, 2015; ZENDEHBAD et al., 2015). C. coli is frequently isolated from pork (QIN et al., 2011; GHIMIRE et al., 2014; WYSOK et al., 2015a; WYSOK et al., 2015b). Although, the lower rates were detected in raw milk and dairy products (WHYTE et al., 2004; YAMAN &ELMALI, 2004; HUSSAIN et al., 2007; BARDON et al., 2011; WIECZOREK & OSEK, 2015; ZENDEHBAD et al., 2015). C. coli is frequently isolated from pork (QIN et al., 2011; GHIMIRE et al., 2014; WYSOK et al., 2015a; WYSOK et al., 2015b). Although, the lower rates were detected in raw milk and dairy products (WHYTE et al., 2004; YAMAN &ELMALI, 2004; HUSSAIN et al., 2007; BIANCHI et al., 2013; MODI et al., 2015; BERTASI et al., 2016; CHRISTIDIS et al., 2016) consistent with our results, YANG et al. (2003), EL-ZAMKAN & ABDEL HAMEED (2016), DI GIANNATALE et al. (2014), KASHOMA et al. (2016) demonstrated a 27.3%, 20%, 17.2%, 13.4% prevalence of Campylobacter in milk samples, respectively. All this emphasizes the importance of raw milk and dairy products as a potential source of Campylobacter. But, Campylobacter was not detected in raw milk in studies from Estonia (KALMUS et al.,2015) and Finland (RUUSUNEN et al. (2013). Negative results may be due to sample contamination.

Table 1 - Primers for identification of Campylobacter isolates and detection of virulence genes.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences (5’ to 3’)</th>
<th>Product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>GGATGACACTTTTCGGAGC</td>
<td>816</td>
<td>Linton et al. (1996)</td>
</tr>
<tr>
<td>Ask (C. coli)</td>
<td>GTATGATTTCACCACACGCGAG</td>
<td>502</td>
<td>Linton et al. (1997)</td>
</tr>
<tr>
<td>glyA (C. lari)</td>
<td>TAGAGGATAGGAAAGAGA</td>
<td>251</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>cj0414 (C. jejuni)</td>
<td>CAAATAAAGITAGAGGTAAGAATGT</td>
<td>161</td>
<td>Wang et al. (1992)</td>
</tr>
<tr>
<td>wlaN</td>
<td>TTAAGGCCAGATATGAAGGTTG</td>
<td>672</td>
<td>Linton et al. (2000)</td>
</tr>
<tr>
<td>cadF</td>
<td>TTTGAAGTTATGTAATATG</td>
<td>400</td>
<td>Konkel et al. (1999)</td>
</tr>
<tr>
<td>cdtA</td>
<td>CACCTGTGATGCAAGCAATC</td>
<td>370</td>
<td>Hickey et al. (2000)</td>
</tr>
</tbody>
</table>
collection, transportation distance and conditions because *Campylobacter* is known to be very sensitive to environmental conditions.

Results obtained from different studies agree with the fact that *C. jejuni* is the most commonly detected *Campylobacter* species and high level contamination (over 50%) with this pathogen is usually recorded in poultry (PEZZOTTI et al., 2003; ELMALI & YAMAN, 2004; BARDOŇ et al., 2011; WYSOK et al. 2015b; ZENDEHBAD et al., 2015). This is because *Campylobacter* species are thermophilic and adapt to poultry body temperature, poultry can easily become contaminated with this pathogen during slaughter and carcass dressing. However, previous studies reported the prevalence of *Campylobacter* in bovine carcass samples ranging from 9.5% (KASHOMA et al., 2016), to 10.5% (WYSOK et al., 2015b) and

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**Figure 1** - Electrophorese image of the isolates. [M: 100 bp DNA marker; 1: Positive control (*Campylobacter coli* ATCC 43478); 2: Negative control (Nuclease free water); 3-4: both 16S rRNA and ask positive isolates (*C. coli*); 5: Positive control (*C. jejuni* ATCC 29428); 6-17: both 16S rRNA and cj0414 positive isolates (*C. jejuni*)].

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**Table 2** - Antimicrobial susceptibility and virulence gene profiles of *C. jejuni* and *C. Coli* isolates from raw milk and cattle slaughterhouse wastewater samples.

<table>
<thead>
<tr>
<th>Source of isolates</th>
<th>Code of isolates</th>
<th>Antimicrobials (Test range, µg/µL)</th>
<th>Virulence genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E (0.016-256)</td>
<td>TE (0.016-256)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>2- C. jejuni</td>
<td>&gt; 256 (R)</td>
<td>8 (R)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>8- C. jejuni</td>
<td>&gt; 256 (R)</td>
<td>12 (R)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>9- C. jejuni</td>
<td>&gt; 256 (R)</td>
<td>6 (R)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>15- C. jejuni</td>
<td>0.125 (S)</td>
<td>0.023 (S)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>18- C. jejuni</td>
<td>&gt; 256 (R)</td>
<td>12 (R)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>21- C. jejuni</td>
<td>0.19 (S)</td>
<td>0.032 (S)</td>
</tr>
<tr>
<td>Wastewater</td>
<td>4- C. coli</td>
<td>&gt; 256 (R)</td>
<td>8 (R)</td>
</tr>
<tr>
<td>Wastewater</td>
<td>5- C. coli</td>
<td>&gt; 256 (R)</td>
<td>6 (R)</td>
</tr>
<tr>
<td>Wastewater</td>
<td>14- C. jejuni</td>
<td>&gt; 256 (R)</td>
<td>16 (R)</td>
</tr>
<tr>
<td>Wastewater</td>
<td>20- C. jejuni</td>
<td>&gt; 256 (R)</td>
<td>96 (R)</td>
</tr>
<tr>
<td>Wastewater</td>
<td>21- C. jejuni</td>
<td>&gt; 256 (R)</td>
<td>16 (R)</td>
</tr>
<tr>
<td>Wastewater</td>
<td>26- C. jejuni</td>
<td>1.5 (S)</td>
<td>0.125 (S)</td>
</tr>
<tr>
<td>Wastewater</td>
<td>27- C. jejuni</td>
<td>&gt; 256 (R)</td>
<td>2 (S)</td>
</tr>
<tr>
<td>Wastewater</td>
<td>28- C. jejuni</td>
<td>0.25 (S)</td>
<td>0.047 (S)</td>
</tr>
</tbody>
</table>

13.1% (WYSOK et al., 2015a). Also, WESLEY et al. (2000) isolated Campylobacter from dairy cattle fecal samples at a level of 39.5%, indicating that milk contamination may originate from fecal contamination during milking.

The prevalence of Campylobacter in wastewater samples in this study (10.2%) is lower than that reported by YANG et al. (2003), but is higher than the results of MOORE et al. (2001) and YAMAN et al. (2005). Our results indicated that wastewaters from cattle slaughterhouses may responsible for environmental contamination with campylobacters in Turkey. Wastewaters from cattle slaughterhouses generally originate from slaughter and rendering units and include blood, intestinal or rumen content. Therefore, contamination should be controlled by improving hygienic conditions at slaughterhouses within the framework of HACCP programs and slaughterhouses must have wastewater treatment plants.

Another part of the study was to evaluate the resistance of C. jejuni and C. coli isolates to some antimicrobials. Resistance to ciprofloxacin was frequently observed in C. coli isolated from wastewater (100%), while lower rates were found in C. jejuni isolates (50%), similar to PEZZOTTI et al. (2003). Resistance to erythromycin (71.4%) was the most common resistance among the isolates different from the results of other authors (CHEN et al., 2010; BARDON et al., 2011; DI GIANNATALE et al., 2014; DUARTE et al., 2014; WIECZOREK & OSEK, 2015; ZENDEHBAD et al., 2015; ZHONG et al., 2016).

As has been observed before (PEZZOTTI et al., 2003; PÉREZ-BOTO et al., 2010; DI GIANNATALE et al., 2014; DUARTE et al., 2014; WIECZOREK & OSEK, 2015), C. coli was more resistant to ciprofloxacin, erythromycin, and tetracycline than C. jejuni in our study. Similar to previous studies (CHEN et al., 2010; QIN et al., 2011; DUARTE et al., 2014; WIECZOREK et al., 2015; ZHONG et al., 2016), multidrug resistance was detected among C. coli and C. jejuni isolates. This result must be taken into account in treatment of campylobacteriosis. Also, the role of C. coli in human campylobacteriosis cannot be ignored due to its multidrug resistance profile.

With regard to the virulence properties of C. jejuni isolates, 75% of them carried virulence-associated genes (cdtA or cadF). Different from our study, WIECZOREK & OSEK (2008) and ROZYNEK et al. (2005) reported cadF gene at most in their isolates. Similary, MARTINEZ et al. (2006), RIPABELLI et al. (2010), GONZALEZ-HEIN et al. (2013), WYSOK et al. (2015a) detected high prevalence of the cdt genes which are responsible for cytotoxin production. Finally, these results also indicated that cdt genes are widespread among human and animal isolates in different countries.

CONCLUSION

C. jejuni was detected as the most prevalent species in the study, followed by C. coli. In addition, more than half of the isolates (57.1%) showed multidrug resistance profiles. As a result, isolation of Campylobacter strains having virulence-associated genes and multidrug resistance from other sources (raw milk, wastewater) could be considered as an important risk both for human and environmental health.

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DECLARATION OF CONFLICT OF INTEREESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS’ CONTRIBUTIONS

The authors contributed equally to the manuscript.

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